A preliminary study of nitrogen fixation in wood-boring-insects and decayed wood

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A PRELIMINARY STUDY OF NITROGEN FIXATION IN WOOD-BORING INSECTS AND DECAYED WOOD

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B.Sc.(Hons.) Cert. Ed.

Being a dissertation presented to the University of Durham in part fulfillment of the requirements for the degree of M.Sc. in Ecology by Advanced Course

September 1982

to

Dominic, Jack & Kate

ABBREVIATIONS AND SYMBOLS

^{o}C	=	degrees Celsius
cm^{3}	=	cubic centimetres
g.	=	gramme
hr.	=	hour
l	=	litre
µg.	=	microgramme
m.m.	=	millimetre
min.	=	minute
nmole	=	nannomole
%	=	percentage
R.H.	=	relative humidity
S.E.	=	standard error
sq. km.	=	square kilometre

ABSTRACT

The possibility that a symbiotic relationship exists between nitrogen fixing bacteria and wood boring insects was investigated using the acetylene reduction assay. Using similar techniques, the presence of nitrogen fixing bacteria in decaying wood was also investigated.

Although no in situ acetylene (nitrogen) reduction could be detected for larvae of the bark beetle Tomicus piniperda or Ips cembrae, 20% of T. piniperda larvae gave a positive result after enrichment, producing 0.048 nmol ethylene $larva^{-1}$ hr^{-1} aerobically and 0.040 nmol ethylene $larva^{-1}$ hr^{-1} anaerobically. Positive acetylene (nitrogen) reduction was also demonstrated in the common furniture beetle larvae Anobium punctatum producing 0.084 nmol ethylene g $larva^{-1}$ hr^{-1} in situ, whilst 72% of larvae tested gave a positive result to the acetylene reduction assay after enrichment.

A positive acetylene (nitrogen) reduction was demonstrated in the termite Reticulitermes santonensis, producing 0.279 nmol ethylene g^{-1} hr^{-1} aerobically and 0.404 nmol ethylene g^{-1} hr^{-1} anaerobically. The levels of acetylene (nitrogen) reduction by the same termite fed on filter paper with no added nitrogen were more than double that found, when fed on a diet of wood. The effect of a diet with differing concentrations of NH_4^+ was to abolish all detectable acetylene (nitrogen) reduction, as did a diet containing high levels of NO_3^-. However a diet containing low levels of NO_3^- had no such inhibitory effect on reduction, producing 0.105 nmol ethylene 50 $termites^{-1}$ hr^{-1} A diet of filter paper impregnated with antibacterial antibiotics also abolished all detectable acetylene reduction. No acetylene reduction could be detected in the wood boring insects Hylotrupes bajulus and Lyctus brunneus.

Samples of decaying wood showed a positive acetylene (nitrogen) reduction in five of eleven sites chosen, with ethylene production ranging from 4.25 to 36.55 nmol g dry wt $^{-1}$ day^{-1} in situ. All these samples gave a positive result after enrichment, in addition to three samples which had given negate results in situ. Different pH optima for acetylene (nitrogen) reduction, were demonstrated for cultures obtained from different decaying wood samples.

CONTENTS

1.1 NITROGEN FIXATION IN DECAYING WOOD AND WOOD BORING INSECTS - A LITERATURE SURVEY

As there appeared to be no single comprehensive reference on this subject, it was considered a useful addition to include a literature survey, as a more general introduction to the subject.

A symbiotic relationship between micro-organisms and certain insects not all specifically wood boring types was discussed by Peklo (1946). Using mainly micro-scopical techniques he examined the eggs and larvae of various insects and discovered that nitrogen fixing organisms exist in both stages, especially in the mycetomes of the larvae. Mycetomes are definite organs, which vary greatly in size and position according to the insect, and are thought to be the site of symbiotic bacteria or yeasts (Imms, 1977). Peklo found bacteria, belonging to the genus Azotobacter, capable of fixing nitrogen, in Aphides, in the corn thrip Limothrips, in the imago of the grain beetle Sitophilus, in the larvae of the moth Sitotroga cerealella, in the larvae of the beetle Anobium paniceum, and in the larvae of two different 'bark-boring' beetles Eccoptogaster rugulosus and Ips (Bostrychus)Typhographus L. Longitudinal microtome sections through the larvae of both species of bark beetles revealed large masses of Azotobacter zoogloeae, especially in the peripheral tissue layers. Peklo stated that these insects would starve for lack of nitrogenous food were it not for the symbiotic Azotobacter, and that insects with well developed mycetomes (aphids Homoptera) fixed free nitrogen much more energetically than Heteroptera, which are devoid of them.

Smith (1948) concluded, however, that as far as aphids were concerned, no nitrogen fixation occurred, when the insects were ground up in a nutrient solution containing glucose and oxaloacetate

incubated at a suitable temperature and pH and any increase in nitrogen, measured by micro-Kjeldahl analysis. He also doubted whether previous work by Peklo (1912), whose morphological studies of the symbiotic micro-organisms of aphids placed them in the Azotobacter group, gave any direct evidence that they could fix nitrogen.

Peklo and Satava (1949) demonstrated an increase in nitrogen content of macerated bark beetles of the genus Ips. They collected large samples of infested bark and isolated the larvae of Ips amitinus L, Ips Typhographus L and Ips chalographus L. The larvae were then crushed and incubated in a nutrient solution and subjected to Kjeldahl estimations. Increases in nitrogen, which they concluded was due to fixation of atmospheric nitrogen, was demonstrated in all species, especially in half grown larvae (three weeks old). They found the results depended on the age of the larvae, so that in very young larvae the adipose tissue containing the symbionts had probably not developed, hence no increase in nitrogen could be demonstrated, and likewise for fully fed, fat, old larvae. In the latter case, the negative result was attributed to the microbial symbionts, which were normally found in the peripheral (adipose) tissue and had migrated into the alimentary canal, and hence digested. They concluded that the results agreed with their cytological considerations (Peklo 1946), and that the increase in nitrogen content of the larvae coincided with the rapid breeding of the insects and so could explain the damage caused by Ips in spruce forests, of Czechoslovakia, at that time.

Hodges et al (1968) found that the southern pine beetle Dendroctonus frontalis Zimm, and its associated micro-organism complex may have caused an increase in the total nitrogen concentration

of pine inner bark. They investigated how the amino-acid pool of inner bark of loblolly pine (Pinus taeda L) was altered by the activity of certain fungi, alone or in combination with the pine beetle. Their results showed increased levels of total nitrogen in infected tissue and they concluded that this could have been due to fixation of atmospheric nitrogen by micro-organisms or by the accumulation in the infected area of nitrogen, translocated from surrounding tissues. They discussed the possibility that in infected tissue a greater source of nitrogenous material was available than in non fungal infected tissue, and this could influence the development of the larvae.

However, it is doubtful that the fungi could have been responsible for nitrogen fixation, as it has since been shown with acetylene reduction techniques and ^{15}N assays, that several eukaryotic micro-organisms, including yeasts and filamentous fungi, described in the literature as nitrogen fixers, are unable to fix nitrogen (Mulder, 1975).

Bridges (1981) reinvestigated the possibility that nitrogen fixing bacteria are associated with bark beetles and influence their nitrogen economy. Using the acetylene reduction technique he demonstrated that nitrogen fixing bacteria could be isolated from three species of beetle, Dendroctonus terebrans, Dendroctonus frontalis and Ips avulsus. A more extended account of Bridges' work is included elsewhere in this dissertation.

The possibility that nitrogen fixation occurs in other insect species was examined by Citernesi et al (1977). In fact they investigated the ecology of nitrogen fixing organisms in the digestive tract of a range of animals. After collection, the animals guts were

dissected out and, either incubated in an enrichment medium or plated out onto a solid enrichment medium to isolate individual colonies. The bacteria were incubated anaerobically before being tested for their nitrogen fixing ability, using the acetylene reduction technique. They demonstrated nitrogen fixing bacteria in two insects, the rose chafer Cetonia aurata, and the coleopteran Tenebrio molitor. As well as arthropods they also investigated other soil animals and were able to isolate pure cultures of nitrogen fixing bacteria from the lumbricid Eisenia foetida, the millipede Glomeris cingulata and the snail Helix aspersa. Their results appear to show the presence of nitrogen fixing micro-organisms in the gastro-enteric cavity, to be a common feature of soil-inhabiting fauna, which feed on decaying organic matter. However the species of micro-organism involved showed no specificity with host animal, and they questioned the factors involved in determining such associations. Within this context, it would have been difficult without further work to state whether the micro-organisms were itinerant or commensal inhabitants of the animals.

Baker (1969) reported that the common furniture beetle Anobium punctatum Degeer, is one of the few species of insects adapted to a diet of sound, dry wood. In a detailed study he investigated the digestion of Scots-pine sapwood, and demonstrated that the proportion of swallowed wood digested was remarkably constant:- 26-29%. Also, only 42% of the total wood protein, which is mostly insoluble, was digested but soluble protein, a minor constituent, was utilized more completely. However the most interesting discovery was that the amount of nitrogen acquired by larvae during their growth was up to two and a half times that provided by the wood, and Baker suggested that Anobium can use fixed

atmospheric nitrogen. It was also probable that Anobium could not reinfest eaten-out wood, unless there had been some change such as that produced by fungal decay, resulting in additional nitrogen being available from the wood. The discrepancy between nitrogen ingestion and assimilation by Anobium larvae, in the presence of excess dietary nitrogen, had not been investigated. The reason was that if too much nitrogen was introduced into the diet, it had an adverse effect on the microbial symbionts of the beetles, causing them to break down. Although it had not been demonstrated, the frass of these larvae could provide the kind of environment where bacteria could flourish, especially since it was also reported that nitrogen fixing bacteria had been demonstrated in the frass of Sirex larvae.

Baker et al (1970) reported on a more complete set of data concerning wood breakdown and nitrogen utilization by Anobium larvae feeding on Scots-pine sapwood. They either infested wood blocks by allowing natural egg laying by adults to occur or by transferring partly grown larvae into predrilled holes. The blocks were then incubated at 22°C, 85% relative humidity for between 18-24 months (for blocks infested by natural egg laying) or 12-18 months (for blocks infested by larval transfer), the wood, frass and insects were then analysed. In both types of experiment the larvae during growth, gained nitrogen from sources other than the wood, so that in the larval-transfer experiments between 43-53% of their total nitrogen came from sources other than the wood, while the comparable figure for the egg laying experiments was 24%. Their results indicated that nitrogen derived from the ingested wood failed to meet the demonstrated nitrogen uptake of the growing larvae, and they

were reluctant to postulate fixation of atmospheric nitrogen by the insect but were unable to account for the results any other way. They suggested that it was unlikely to be yeast-like symbionts which live in the mycetomes of this insect, but more likely to be bacteria or possibly Actinomycetes living within the complex of wood/frass/insect.

Hungate (1941) investigated the possibility of nitrogen fixation within termite colonies. Cultures of Zootermopsis termites were started with weighed amounts of wood and termites, samples of which were analysed for nitrogen. The wood used was Monteray pine, Pinus radiata, and the cultures were maintained in a container for variable periods of time to a maximum of 25 months, after which further nitrogen analysis was carried out. The chief feature of the results was the general failure to obtain increases in nitrogen within the colonies, and hence they failed to demonstrate any significant fixation of atmospheric nitrogen.

Further experiments by Hungate on the nitrogen content of termite-infested wood revealed significantly higher levels of nitrogen compared with non-infested heartwood and sapwood. This was explained by reference to the nitrogen coming into the wood from the closely adjacent soil, since previous experiments had discounted nitrogen fixation. In another experiment Zootermopsis nevadensis was set up as a starting culture in Monteray pine, using either heartwood or sapwood, and incubated for thirteen months. On analysis it was shown that the % of nitrogen in the wood more than doubled and this was accounted for by the presence of fungi, which increased the concentration of nitrogen in the wood by decomposing the carbohydrate constituents and supposedly bringing in nitrogen from the soil. There

was no indication of fixation of atmospheric nitrogen, and it was concluded overall that nitrogen used for the growth of the termites was brought into the wood by fungi, presumably as part of a fungus body, although the type of fungus involved was also an important factor, as the action of some was more favourable than others.

Further experiments on Kalotermes synderi cultured on homogenized wood again failed to reveal any indication of nitrogen fixation, and were considered to be carried out under more carefully controlled conditions than any of the others reported in the paper. A concluding set of experiments with the genus Reticulitermes, again backed up the results with Zootermopsis, the increases in nitrogen being attributable to the activities of certain fungi.

The rôle of symbiotic bacteria in the nitrogen economy of termites and other herbivorous insects and the possibility of fixation of atmospheric nitrogen, was also discounted by Wigglesworth (1965).

Benemann (1973) reinvestigated the question of nitrogen fixation in termites using the acetylene reduction assay. Different castes of Kalotermes minor were collected from a pine post and incubated with wood in 7 cm^3 serum flasks under different atmospheric conditions. They were then assayed for acetylene reduction using gas chromatography, following the introduction of 16.5% acetylene. The soldier and reproductive castes fixed little or no nitrogen, but the worker castes all fixed nitrogen in varying levels under the different atmospheric conditions. It was shown that fixation could proceed in aerobic, anaerobic or microaerophilic conditions (20% oxygen 80% argon). Variations in fixation rates were observed between different batches, and the cause explained as possibly being related to the workers age. Further experiments with other termites, Cryptotermes brevis and

Zootermopsis angusticollis, also revealed nitrogen fixation but at much lower rates. (10 μg of N fixed month^{-1} gram wet weight^{-1} compared to a maximum of 566 μg N fixed month^{-1} gram wet weight^{-1} in Kalotermes.) It was concluded that nitrogen fixation must be a major source of nitrogen for at least some termites under some circumstances, although it was noted that even at the highest fixation rates, 30 months would be required to double the nitrogen content of the termites. Further studies would reveal whether nitrogen fixation was the only source of nitrogen for these wood eating insects. The variation and magnitude of the fixation rates probably explained the failure to detect nitrogen fixation in previous work (Hungate, 1941).

Breznak et al (1973) also used the acetylene reduction technique to investigate nitrogen fixation in the subterranean termite Coptotermes formosanus Shiraki, using techniques similar to those outlined above (Benemann, 1973). They were able to demonstrate fixation in isolated termite guts, suggesting that micro-organisms present in the gut were responsible for the fixation.

Further experiments using different levels of ammonium and/or nitrate nitrogen, fed to the termites by impregnation on filter paper, also revealed variations in the fixation rates, as did experiments involving filter paper impregnated with antibiotics. In the latter type of experiment, all detectable nitrogen fixation was abolished, which was presumably due to a loss of bacterial activity caused by the antibacterial antibiotics. Further experiments revealed that the nitrogen fixing activity in these termites rapidly responded to changes in dietary N content. Thus within 24 hours the nitrogen fixing activity of their gut symbionts could be utilized to supply the

termites, quickly, with combined nitrogen, should the dietary level suddenly drop. Using similar techniques they also assayed representatives of seventeen genera of insects, including cockroaches, ants, beetles, aphids, fruit flies and bugs, but failed to detect nitrogen fixing activity in any of them.

Later work by Potrikus and Breznak (1977) showed that the bacterium Enterobacter agglomerans was responsible for fixing nitrogen in the gut of certain wood eating termites.

French et al (1976) examined three worker caste species of Australian termites, Coptotermes lacteus Frogatt, Mastotermes darwiniensis Frogatt and Nasutitermes exitiosus Hill ,using the acetylene reduction technique, for their ability to fix nitrogen. In all cases, they demonstrated positive results on both live workers and on bacteria which were isolated from the hind guts by streaking out their contents on N-free agar. The activity measured varied between species, being highest in M. darwiniensis and very low in C. lacteus, which agreed with data from the related termite C. formosanus outlined above (Breznak et al, 1973). The isolated bacteria were all gram negative anaerobic rods, and were all identified as Citrobacter freundii (Braak) Werkman and Gillen, and formed the first record of nitrogen fixation by this species.

Evidence for nitrogen fixation, using the acetylene reduction assay, has also been reported in a wood eating cockroach Cryptocercus punctulatus Scudder (Breznak et al, 1974).

Nitrogen fixation in Wood

In contrast with herbaceous tissues which contain 1-5% N by weight, woody plant tissue usually contains only 0.03-0.10% N, and has a C:N ratio of (350-500):1 and in some species is even higher, 1250:1, e.g. in the highly decay-susceptible heartwood of sitka spruce (Cowling et al, 1966). Therefore for most saprophytic organisms the

very high C:N ratio of woody tissue would support little growth; but wood destroying fungi apparently are well adapted to wood as a substance and still produce large fruiting structures and enormous numbers of spores, despite its meagre N content. To account for the success of the wood-destroying fungi, Cowling et al (1966) suggested three possible mechanisms: (i) physiological adaptations resulting in preferential allocation of available N to metabolically active substances and pathways, highly efficient in the utilization of wood constituents; (ii) reuse of available N by a dynamic and continuous system of autolysis of less active hyphae and reuse of nitrogenous constituents by more active regions of mycelia without significant loss; (iii) utilization of N from sources outside the wood, so that the fungi would not be dependent upon N in wood as their sole source, an example would be that supplied by fixation of atmospheric nitrogen. It was then suggested that tests for possible fixation by wood-destroying fungi be carried out using ^{15}N, to check this hypothesis.

Previous work by Klingström et al (1963) on the wood destroying fungus Merulius lacrymans had investigated the possibilities of nitrogen fixation. They incubated cubes or shavings from pine sapwood in flasks inoculated with the fungus, together with suitable controls. At intervals of 2, 4, 6 and eight months they compared the nitrogen contents of inoculated flasks against the controls, using Kjeldahl analysis. However their work failed to reveal any significant differences between the two sets of flasks and hence the possibility of nitrogen fixation was discounted.

Millbank (1969) carried out a re-appraisal of the ability of yeasts and moulds to fix atmospheric nitrogen. All were isolates from soil, and were tested using both the heavy nitrogen (^{15}N) and acetylene reduction techniques. No fixation was observed in any of the organisms.

Seidler et al (1972) reinvestigated the question of nitrogen fixation in decayed wood, but concentrated on the bacterial isolates cultured from the wood. They carried out a study of decay in living white fir trees (Abies concolor (Gord. & Glend)) growing in a forest in south western Oregon, which showed evidence of fungal decay caused either by the genera Echinodontium or Phellinus. They aseptically collected samples of decayed wood and incubated them in different types of growth and enrichment media until bacterial growth was observed. The cultures were then tested for nitrogen fixing abilities using the acetylene reduction technique. Of the one hundred and fifty four cultures isolated, forty showed rapid rates of acetylene reduction and hence had the ability to fix nitrogen. Of these 43% were isolated from wood which was discoloured wet looking and adjacent to bright wood, 12% from wood in advanced decay and 45% from wood in incipient decay. Attempts were made to identify the nitrogen fixing isolates but were not very successful. It was considered to be the first report of nitrogen fixing bacteria in decays on living trees. The significance of this discovery would mean that nitrogen fixation could definitely serve as an auxiliary source of nitrogen for organisms active in causing decomposition, and hence play an important rôle in regulating the rate of wood decomposition in forest ecosystems.

Cornaby et al (1973) used the acetylene reduction technique to investigate the possibility of nitrogen fixation in decaying logs of American chestnut, Castanea dentata (Marsh) Borkh. These were obtained from a mixed-hardwood forest in North Carolina, and replicate samples of wood were removed from sites across a vertical cross-section of logs in different stages of decay. The samples were placed in 130 cm^3 jars and analysed for nitrogen reduction activity using the acetylene reduction assay, by incubating with 20 cm^3 of acetylene for

24 hours. The results showed nitrogen fixation for both aerobically and anaerobically cultured samples, of the same magnitude as that recorded in total litter, and higher than that of soil taken from the same site, and calculated an annual fixation rate in logs of 0.887 kg N_2 fixed, hectare^{-1}, year^{-1}. Thus it was concluded that nitrogen fixation could play an important rôle in the rates of wood decomposition.

Sharp et al (1973) also investigated whether nitrogen fixing bacteria could penetrate wood and participate in its deterioration. Sets of wood veneers and of cubes of similar size were taken from - lime, beech, oak and Scots-pine and buried in 200 g.portions of soil, which in some cases was augmented with glucose. In certain of the samples, some of the transverse faces were sealed by a silicone adhesive. All samples were incubated for 20 days at 25°C except the lime which completed 40 days at 28°C. On removal from the soil the samples were washed and sandpapered to remove superficial growth, before being cut into slivers and examined using the acetylene reduction assay.

All the woods examined permitted the penetration of micro-organisms capable of fixing nitrogen. This was the first report of such activity in wood with ground contact. The addition of glucose greatly stimulated the acetylene reduction, but it was not always consistent, probably being affected by variable soil conditions.

Sharp (1974) repeated some of the experiments on veneers of beech, oak and Scots-pine and again was able to demonstrate acetylene reduction in all samples. The levels of N_2 fixed ranged from 0.012 to 0.031n mol of N_2 hr.$^{-1}$, day^{-1} (nitrogen fixed was assumed to be equivalent to $\frac{1}{3}$ of the amount of molecules of ethylene formed). It was

considered that the question of whether the fixed nitrogen was available for fungal growth, needed to be established. Since many species particularly pioneer colonizers, appeared to grow in conditions of low N it was thought that fixed nitrogen might only be important when the total amount present was extremely small.

Aho et al (1974) provided more detailed information on the character and distribution of N_2 fixing bacteria in decaying white fir trees and presented quantitative data on the capability of certain bacterial isolates from decaying wood to fix N_2. They collected samples from living white firs which showed evidence of decay, using 62 different sampling sites over a period of two years. Samples of the decayed wood were aseptically collected, and the bacteria isolated, purified and identified, using normal cultural and diagnostic tests. The cultures were also tested for their ability to fix nitrogen using the acetylene reduction assay. Of the isolates tested, 52% of the gram negative fermentative bacteria reduced acetylene anaerobically, and did so at levels similar to those reported for other nitrogen fixing bacteria. Although higher numbers of the fermentative and nitrogen fixing bacteria were associated with early and incipient stages of decay, a higher percentage of the fermentative isolates from advanced decay fixed nitrogen. The N_2-fixing bacteria isolated were associated with four major decay fungi, Echinodontium tinctorium, Pholiota adiposa, Phellinus chrysoloma and Hericium abietis, which accounted for more than 90% of all decay in living white firs in the area studied. No N_2 fixing bacteria were isolated from decay caused by Fomitopsis annosa Karst or Armillariella mellea Quél. These so called "pioneer fungi" were known to be capable of invading wood in living trees, before colonization

by bacteria and non-hymenomycetous fungi. Counting techniques (most probable number)revealed counts of 3.5×10^5 of nitrogen fixing colonies cm^{3-1} of expressed sap from early decay sites, and it was thought that these high concentrations indicated they probably play an important rôle in heartwood decay. It was also suggested that a mutualistic relation existed between bacteria and wood decay fungi, where the bacteria benefit from cellulose and hemicellulose decomposition and the formation of organic acids by the fungi. In return the bacteria provide the nitrogen for the decay fungi, which is needed to provide for the synthesis of wood digesting enzymes in wood with a meagre N content.

Of the 130 isolates tested only 20 were found to be biochemically typical of previously described species; twelve cultures were identified as anaerogenic Enterobacter agglomerans, six were E. aerogenes and two were Klebsiella pneumoniae.

Sharp (1975) investigated the nitrogen fixing activity, as measured by the acetylene reduction technique, of various micro-organisms growing on wood, under various conditions. Centimetre cubes or thin veneers of beech wood were inoculated with soil and then either supplemented with antibiotics and incubated for 40 days at 25°C or were perfused with water and incubated at different temperatures, or were adjusted to different pH readings and incubated for 30 days at 25°C. Additional experiments involved investigating the influence of wood preservatives by immersing centimetre cubes of beech and Scots-pine in a preservative for one hour and then leaving them buried in soil for 30 days at 25°C. An attempt was also made to demonstrate if $^{15}N_2$ incorporated into deteriorating wood, would be transferred to invading fungi. The results of the antibiotic

experiment suggested that the presence of active fungi reduced the activity of nitrogen fixing bacteria and could have arisen from any of the interactions micro-organisms display. The optimum temperature for nitrogen fixing activity was found to be 35°C, and the optimum pH to be 3.5. The demonstration of nitrogen fixation in only a few preserved woods, indicated a method for evaluating preservatives and other toxicants. No evidence was found, with the technique employed, that fixed N_2 could be passed to fungi. This failure was explained by the likelihood of slow rates of N_2 translocation in hyphae, together with the comparative insensitivity of the technique and the short incubation allowed. Since both nitrogen fixation and incorporation of ^{15}N into wood occurred, it was considered that some of the fixed N_2 would eventually be taken up by the non-fixing fungal colonists, since its presence in water washings of the wood suggested it was not in a labile and unavailable form.

Larsen et al (1978) investigated nitrogen fixation associated with wood decayed by some common fungi in Western Montana. Small pieces of wood, from a decayed area, were analysed by the acetylene reduction technique for evidence of nitrogen fixation. The wood was also incubated in malt agar and the resulting fungal growth noted and the species identified. Their results indicated that there was an increased capability for supporting nitrogen fixation with decay progression.

There was a significant difference between nitrogen fixation rates related to the type of rot observed with the highest rates being found in the brown rot systems. Since brown decayed wood had previously been shown to be an important ecological niche for

formation of mycorrhizae, it was suggested that a strong nutritional relationship might exist between mycorrhizae and nitrogen fixation. It was concluded that the rates and quantities of nitrogen fixation in decaying woody residues were important components of the nitrogen cycle in the areas studied, and that woody residues should be viewed as a substrate with important and unique biological functions.

1.2 INTRODUCTION

The occurrence and functioning of nitrogen-fixing bacteria in different habitats, is widespread. Their activity has been reported in niches as diverse as paper mill process waters (Neilson et al, 1976) to the human intestine (Bergessen et al, 1973). One of the first studies of a symbiotic relationship between nitrogen-fixing bacteria and insects, was reported for aphids (Peklo, 1912). Although their diet can be low in protein, the evidence for nitrogen fixation by herbivorous insects has been discounted (Hungate, 1941; Smith, 1948; Wigglesworth, 1965). However the acetylene reduction assay for nitrogen fixation provided a method for re-investigating the problem. The assay depends on the ability of the nitrogen fixing enzyme, nitrogenase, to reduce acetylene to ethylene and so provide a reliable and sensitive measure of fixation potential (Hardy et al, 1968).

Since then, evidence for nitrogen fixation in insects has been found with the acetylene reduction assay for several species of termites (Benemann, 1973; Breznak et al, 1973; French et al, 1976; Potrikus et al, 1977), for two species of soil insect (Citernesi et al, 1977), for a wood eating cockroach (Breznak et al, 1974), and for three species of bark beetle (Bridges, 1981). The present study was carried out to investigate whether such a symbiotic relationship could be demonstrated in other wood-boring insects, making use of the acetylene reduction assay.

The species chosen were obtained from a variety of habitats and included two species of bark-boring beetles, Tomicus piniperda L., a pine shoot beetle found throughout the British Isles and Ips cembrae Heer, a beetle found only in Scotland. The presence of nitrogen fixing bacteria in the common furniture beetle Anobium punctatum Degeer has been suggested (Baker, 1969; Baker et al, 1970),

and therefore provided a basis for further examination in this study. In addition the house longhorn beetle (Hylotrupes bajulus L.), the powder post beetle (Lyctus brunneus L.) and a species of termite Reticulitermes santonensis L., were also investigated.

A concurrent preliminary small scale study was also carried out to investigate the possibility of nitrogen fixing micro-organisms inhabiting dead or decaying wood. The C:N ratio of woody plant tissue is about (350-500):1 and can be even higher, and it has been suggested that nitrogen fixation provides additional nitrogen for wood-destroying fungi to utilize wood as a substrate (Cowling et al, 1966). The presence of nitrogen fixing micro-organisms in decaying wood has been reported for different tree species (Seidler et al, 1972; Cornaby et al, 1973; Sharp et al, 1973; Aho et al, 1974; Sharp 1975; Larsen et al, 1978). In this present study, further samples of decayed wood from different trees was examined using the acetylene reduction assay, and experiments were conducted to investigate whether the micro-organisms involved were influenced by pH.

1.3 Wood-boring insects

1.3.1 Anobium punctatum

The common furniture beetle is a small brown insect varying in length from 2.5 to 6.00 mm. It can fly but those usually found flying are males, the females preferring to hide in old flight-holes. The female lays eggs in small groups of 2, 3 or 4, wedged in cracks, crevices or joints of unpainted and unpolished wood, or sometimes just pushed inside old flight-holes. On emergence from the egg the larva commences to bore into the wood and will spend the whole of its larval life inside the wood. It grows to about 7 mm in length and is greyish

Wood infested with Anobium punctatum showing the characteristic exit or flight holes.

white in colour, being strongly arched or 'crescentric' in shape. The larva mostly tunnels up and down the grain, but from time to time will cross over into a different growth ring. The tunnel or gallery is loosely filled with powdery bore dust or frass, consisting of rejected wood fibres and faecel pellets. The number of larval moults is unclear, but when mature it constructs a pupal chamber directly beneath the wood surface, inside which it then pupates. The pupal stage is said to last from 6 to 8 weeks after which the adult bites its way out of the wood, making the characteristic exit or flight-hole (see photograph). The life cycle probably averages between three and four years, and never less than two.

It is considered to be one of the most important insect pests found in buildings in Britain, and an active infestation by this insect is thought to occur in at least 50% of dwellings in Britain (Hicken, 1964). It is a common insect out of doors where it is found attacking dead wood still attached to a living tree, such as branch scars or where bark has been rubbed off. The attacked wood is usually hardwood such as oak or fruit-tree, yet indoors it is mainly a softwood pest, found in rafters, joists, and flooring or joinery of the ground and first floor. However its name denotes that it was formerly more important as a pest of furniture, which is mainly hardwood.

1.3.2 Hylotrupes bajulus

The house longhorn beetle is a pest of softwood, important only in a relatively small area of north-west Surrey where 50% of all buildings were thought to be infested (Hicken, 1964). The eggs are laid in clutches in crevices in the wood and hatch out into shining ivory-white larvae. These tunnel through the outer sapwood and

Exposed pine log showing the characteristic mozaic of feeding tunnels, after bark removal, made by Tomicus piniperda larvae.

grow fast, but as they tunnel deeper into the wood, the rate of growth slows down, they can grow to a length of 24 mm. The larval galleries are oval in cross-section and are filled with frass, consisting of rejected wood fragments and cylindrical faecel pellets. The pupal stage lasts only about 20 days and the adult bites its way from the pupal chamber, leaving an oval flight hole in the wood. The life cycle can last from 3 to 10 years and the maximum recorded is 32 years; the greatest part of the cycle is represented by the larval stage.

1.3.3 Lyctus brunneus

The powder post beetle is a pest of the sapwood of certain wide pored hard woods, e.g. oak and ash. It is a pest of furniture and hardwood floors or fittings but has been reduced in importance due to large scale eradication procedures in timber-yards. On hatching the larvae form irregular tunnels as they cut through the wood, and reach about 5 mm when fully grown. It pupates in a chamber beneath the wood surface, before eating through the surface and flying away as the adult.

1.3.4 Reticulitermes santonensis

R. Santonensis is a termite, normally found in warm temperate or tropical areas of the world; there are no termite species native to the British Isles. Members of the genus Reticulitermes construct a very diffuse nest and live in the ground and infest wood indirectly through the soil. They can cause excessive damage to any woodwork of buildings in contact with the ground and often construct covered passage-ways of earth or faecel material, which enable them to work concealed and surrounded by the requisite humidity. R. Santonensis, like all termites, is polymorphic and lives socially in large

Exposed larch log showing Ips cembrae

at various stages of development

communities. Individuals are divisible into reproductive and sterile forms, each of which comprises a number of castes, i.e. "morphologically and functionally distinct terminal forms incapable of further transformation" (Wigglesworth, 1964). In this present study, only representatives of the worker caste were examined.

1.3.5 Tomicus piniperda

T. piniperda is a pine shoot beetle which occurs throughout Britain and causes damage to British pinewoods, especially the Scots-pine (Pinus sylvestris). Adults over winter in hollowed out pine shoots or in short tunnels in and under the bark at the base of the trees. They emerge and breed in spring, usually in thick well fissured bark. The larvae are white, wrinkled and legless, with a well developed brown head and strong biting mouth parts. They burrow into the bark and cambial layer, at first at right angles to the mother gallery in which the eggs were originally laid, but later more haphazardly forming a characteristic mozaic of feeding tunnels (see photograph). They pass through four instars and then pupate, after which the adult emerges from the wood by gnawing neat, round holes through the bark. The adults then feed by boring up the centre of pine shoots, usually those of the current years growth. The beetles, though apparently ready to start brood production, rarely do so until the following spring.

The major damage to the tree is caused by the adult feeding, which causes "shoot pruning", which when severe enough can permanently damage the crowns of affected trees (Davies & King, 1977).

1.3.6 Ips cembrae

This bark beetle arrived in Britain from Europe after the last war, and is now established in most of Scotland, but is not found south of the Tweed Valley. It normally breeds in larch logs and

material down to 4 cm diameter is suitable, but it will also attack and kill standing larch which are already debilated and weakened by drought. The adults over winter in the ground or in logs or as pupae in logs. The males form long egg galleries in logs or standing trees prior to mating and the eggs hatch into larvae which feed on the inner bark mostly in the phloem tissue (Balogun, 1970), forming characteristic feeding tunnel mozaics (see photograph). They pass through three instars before pupating and emerging as adults in July or August. The adults can infest further logs or fly up to the crown of larch trees and feed by tunnelling into shoots, 1 cm in diameter, weakening them so that they are easily broken off by the wind.

2. MATERIALS AND METHODS

2.1 Collection of samples

2.1.1 Insects

Samples of the pine shoot beetle Tomicus piniperda, were collected from infested pine logs in Hamsterly Forest, County Durham in May, 1982. This forest covers some 18 sq. km. and is situated between the rivers Wear and Tees about 21 km. west of the town of Bishop Auckland (National Grid NZ 1131). Cut pine logs known to be infested with the larvae of T. piniperda were placed in large polythene bags and transported back to the laboratory, where they were stored outside until required.

Samples of Ips cembrae were collected from infested larch logs, which had been recently felled, in Glentress Forest, The Borders, in July, 1982. This is a much smaller forest situated 3 km. east of Peebles in the Tweed Valley (National Grid NT 2742). Again, cut logs were placed in polythene bags and transported back to the laboratory, to be stored outside until required. As the logs were collected in July, they contained all stages of the beetle, i.e. larva, pupa and adult.

Anobium punctatum larvae were obtained from the research division of Rentokil Ltd., East Grinstead. Rentokil is a nationwide industrial concern, which specializes in the treatment of woodworm, and hence maintains and cultures Anobium larvae. The larvae were kept at 22°C and 70% Relative Humidity in individual small tubes (if kept together they may be found to have injured one another by biting). Samples of A. punctatum larvae were also obtained from the Building Research Laboratory, Princes Risborough. These samples were kept inside young hazel stems, which had originally been infested by adults laying eggs inside the wood.

Samples of Reticulitermes santonensis, were also obtained from Rentokil (permanent culturing requires a Home Office licence). These were kept in contact with damp potting compost (John Innes No.2) with small wood chips, at about 25°C, and 70% Relative Humidity.

Hylotrupes bajulus and Lyctus brunneus larvae were also obtained from the Building Research Laboratory. Hylotrupes larvae were kept in individual small tubes whereas the Lyctus larvae were kept in either pressed cellulose powder blocks or in square oak blocks of wood, all were kept at 25°C and 70% Relative Humidity until required.

2.1.2 Wood

Samples of decayed wood were collected from various sites close to the University. These included samples of Acer (sycamore) and Quercus (oak) from the Deerness Valley area of Durham and Salix (willow), Acer, Fagus (beech) and Fraxinus (ash) from sites along both the north and south banks of the river Wear, adjacent to Prebens Bridge, Durham City. In all cases the wood was removed from decaying tree trunks, as nearly aseptically as possible, and immediately transferred to individual polythene bags, for transport back to the laboratory. Usually three sample sites were chosen for each decay area, and in one willow sample, wood was sampled from a site infected with the basidiomycete Dacryomyces stillatus. Samples which were tested almost immediately were stored at room temperature, though some samples were stored at 3°C until required.

2.2 Preparation of samples for acetylene reduction assay

2.2.1 Insects

In all cases, the insects were surface sterilized to remove any possible contaminating micro-organisms. This involved brief immersion in an improved Whites sterilizing solution which consisted of -

1.0 g. $HgCl_2$ l^{-1}

6.5 g. NaCl l^{-1}

1.25 cm^3 HCl l^{-1}

250 cm^3 95% ethanol

750 cm^3 sterile distilled water

which has been shown to reduce contamination to as low as 3% (Barras, 1972). To investigate the possibility of in situ acetylene reduction, single larva (H. bajulus), or groups of larvae (A. punctatum, I. cembrae T. piniperda, L. brunneus) or groups of fifty adult workers (R. santonensis) were placed in sterile 7 cm^3 serum bottles fitted with screw tops and rubber liners. In the case of I. cembrae groups of ten larvae, pupae and adults were each investigated. In all cases replicates were set up in air and flushed with nitrogen to create anaerobic conditions so that both aerobic and anaerobic in situ acetylene reduction could be investigated.

Attempts were also made to isolate nitrogen-fixing bacteria by enrichment cultures for both species of bark beetle, A. punctatum, L. brunneus and H. bajulus. The enrichment culture medium used was prepared from two solutions (Neilson et al, 1976; Bridges, 1981). Solution 1 contained -

6.3 g. K_2HPO_4

1.9 g. $Na\ H_2PO_4 . H_2O$

0.1 g. $Mg\ SO4 . 7H_2O$

0.008 g. Na_2MoO_4

0.008 g. ferric citrate

0.5 g. sodium thioglycollate

0.2 g. yeast extract

0.001 g. resazurin

950 cm^3 distilled water

Solution 2 contained 50 cm^3 of a 20% glucose solution.

Solution 1 was autoclaved without the K_2HPO_4 (which was autoclaved separately to avoid precipitation problems). These were then both added to the glucose solution, which had also been autoclaved separately, to form the final medium.

Single larvae (pupae and adults as well, for I. cembrae) were placed in a sterile 7 cm^3 serum bottle, containing 1 cm^3 of enrichment medium, and crushed with a clean glass rod to produce a macerate. Replicate samples were always set up and flushed with nitrogen to create anaerobic conditions, and the macerates were then incubated at 25°C for 3 days, before testing for acetylene reducing activity.

Samples of insect frass (debris or excrement produced by larvae) were also collected from the two species of bark beetle. These were also tested for acetylene reducing activity by placing one gram aliquots in 7 cm^3 sterile serum bottles and testing as such, or adding 2 cm^3 enrichment medium and incubating as above, before testing.

Samples of inner bark next to the galleries of T. piniperda and I. cembrae were also checked for aerobic and anaerobic acetylene reduction activity with and without enrichment, as was the wood in the termite colony. Any enrichments which were positive, were subcultured into fresh medium, and re-tested for acetylene reduction.

2.2.1.1 Additional experiments with R. santonensis

Since some termites can live on a diet of cellulose filter paper (Hungate, 1941) it was decided to investigate the influence of a diet rich in combined nitrogen, on the acetylene reduction assay. The synthesis of the enzyme nitrogenase (responsible for nitrogen fixation and hence acetylene reduction) is known to be repressed when NH_4^+ is present (Burris, 1979).

Groups of 50 R. santonensis workers were placed in glass Petri dishes, each containing a 7 cm circle of Whatman No.2 cellulose filter paper. Six experiments were carried out with one group having their filter paper impregnated with 0.6 cm^3 of an aqueous trace salt solution containing basal salts. This had the composition as mg. per 100 cm^3 distilled water of

30 KH_2PO_4

20 $MgSO_4.7H_2O$

2 $MnSO_4.H_2O$

5 $CaCl_2.2H_2O$

0.2 $FeCl_3$

pH adjusted to 7.0 with NaOH

A second and third group had the same salt mixture but with the addition of 0.8 mg $(NH_4)_2SO_4$ and 8.0 mg $(NH_4)_2SO_4$ respectively, whilst a fourth and fifth group had 1.2 mg KNO_3 and 12 mg KNO_3, respectively. One further sixth group were given the same basal salt mixture but in addition various antibacterial antibiotics had been added, to investigate their effect on the microbial population. These antibiotics, tetracycline, 480 µg; pencillin, 540 µg; chloramphenicol, 600 µg; and streptomycin sulphate, 600 µg per 100 cm^3. The termites were then incubated with the moist filters at 25°C for ten days, before being tested for acetylene reduction activity.

2.2.2 Wood

Samples of both decayed and sound wood were removed aseptically using sterilized scissors, a scalpel or a chisel, and cut into veneers of approximately 30 mm x 20 mm x 2 mm. Care was exercised not to incorporate woody material from the outer exposed portions. They were

weighed, and their volume determined by water displacement for groups of ten veneers. Each sample was also analysed for pH, by washing portions of wood with water and checking the pH using a pH meter (model 7020, Electronic Instruments Ltd., Surrey, England). Samples of ten veneers were then placed in a sterile 130 cm^3 conical flask with 40 cm^3 of sterile distilled water and fitted with a No.41 Suba-seal closure, and analysed for in situ acetylene reduction activity. Samples of wood were also enriched with 40 cm^3 of culture medium, as used during the insect experiments and incubated at 25°C for four days in the dark (to preclude the possibility of growth and subsequent acetylene reduction by any blue green algae in the wood). Following incubation samples were then tested for acetylene reduction.

Following analysis for ethylene production, any positive enrichment cultures were retained and re-examined to investigate the influence of pH on nitrogen (acetylene) reducing activity. Exactly 1 cm^3 of enriched culture was placed in a 7 cm^3 sterile serum bottle containing 1 cm^3 of water adjusted to a pH between 2-8 with phosphate-citrate buffer (Hale, 1965) and left at 25°C for seven days before testing for acetylene-reduction activity. Replicate samples for each pH were tested.

2.3 Testing for acetylene reduction

This technique is by far the most useful and widely applied method for estimation of nitrogen fixation (Hardy et al, 1973). It is at least a thousand times more sensitive than $^{15}N_2$ uptake and considerably faster and cheaper than mass spectrometric analysis (Mague, 1978). The advantages of the technique are reviewed by Hardy et al (1973). The generally accepted method of quantifying rates of acetylene reduction obtained experimentally is to measure the total nitrogen in the sample

and express the result as a fraction of this value. However this was not possible in this preliminary study, due to its short term nature.

The two types of incubating chambers used were either 7 cm^3 serum bottles fitted with screw tops and rubber liners or 130 cm^3 conical flasks fitted with No.41 Suba-seal closures. To prevent possible contamination from previous assays (Waughman, 1971) the liners and closures were aired for at least two weeks after completion of an experiment, before being used again.

To carry out the assay in a serum bottle, 1 cm^3 of acetylene, which gave a concentration of 16% (by volume) was injected through the rubber stopper lining, using a Gillette Sabre disposable syringe. When using a 130 cm^3 flask, the amount of acetylene added was also calculated to give a concentration of 16%. After injection the bottle or flask was vented to adjust the pressure of the gas phase inside the container. Acetylene used was conveyed in a football bladder fitted with a rubber tube, into which a needle could be inserted and a bung. The bottle or flask was then incubated for twenty four hours at 25°C. At the end of the incubation period, samples containing enrichment medium were shaken to allow maximum amount of gas to be released into the gas phase of the container.

Ethylene was estimated by withdrawing a 1 cm^3 sample of gas from the container with a disposable Sabre syringe and injecting directly into a Varian Aerograph Series 1400 gas chromatograph. The apparatus used, was fitted with a stainless steel column (1800 mm x 3.2 mm) packed with Poropak R and maintained at 98°C. Nitrogen was used as the carrier gas, at a flow rate of 45 cm^3 min^{-1}.

The peaks for ethylene values obtained on the chromatograph were

converted into volumes of ethylene (nannomoles), by relation to a standard peak obtained from a known volume of high purity ethylene (B.D.H. Chemicals Ltd.), prepared using a Hamilton gas syringe. A known volume of ethylene was injected into a 1 litre volumetric flask fitted with a Suba-seal, whose volume was accurately known. The ethylene was allowed to mix with the air (glass beads facilitated mixing of the gases when the flask was shaken). Four 1 cm^3 samples of gas were then taken from the flask, injected into the chromatographic apparatus and the resulting ethylene peaks read off. The mean of the values was then used as the standard peak. The chromatograph was calibrated every time gas samples were analysed. In addition ethylene contamination of the acetylene was determined in each experiment by including controls containing no samples. Controls were also included to check on any ethylene production by the samples, particularly the wood, by not injecting with any acetylene. Any ethylene contamination was allowed for in the final calculations.

3. RESULTS

3.1.1 Tomicus piniperda larvae

There was no in situ acetylene reduction for any larva, assayed aerobically or anaerobically.

However on culturing larvae in enrichment media, the results were:

Table 1. Ethylene production by Tomicus piniperda larvae after enrichment

	number cultured	positive C_2H_2 reduction	% positive	mean C_2H_4 production nmol larva^{-1} hour^{-1}		standard error of mean
aerobic	51	6	12	0.048	±	0.020
anaerobic	42	12	29	0.040	±	0.019

3.1.2 Tomicus piniperda frass and gallery bark

Again no in situ acetylene reduction could be demonstrated.

Culturing samples in enrichment media, gave the following results:

Table 2. Ethylene production by Tomicus piniperda frass and gallery bark after enrichment

	number cultured	positive C_2H_2 reduction	% positive	mean C_2H_4 production nmol g^{-1} hour^{-1}		standard error of mean
frass:						
aerobic	5	4	80	0.023	±	0.008
anaerobic	5	3	60	0.056	±	0.007
bark:						
aerobic	9	4	44	0.015	±	0.004

3.2 Ips cembrae

No acetylene reduction could be demonstrated for any larva, pupa or adult either in situ, or after enrichment. A similar negative result was obtained for samples of frass and gallery bark.

In all 44 larvae, 30 pupae and 30 adults were tested aerobically and 42 larvae, 30 pupae and 30 adults tested anaerobically, in situ and after enrichment.

3.3 Anobium punctatum larvae

In situ acetylene reduction gave positive results in four of the five experiments.

Table 3. Ethylene production by Anobium punctatum larvae in situ

	C_2H_4 nmol $hour^{-1}$	
experiment	Per 50 larvae	Per g. larvae
1	0.035	0.075
2	0.033	0.077
3	0.041	0.105
4	0.031	0.079
5*	0.000	0.000

On culturing individual larva in enrichment media, anaerobically the results were:

Table 4. Ethylene production by Anobium punctatum larvae after enrichment

number cultured	positive C_2H_2 reduction	% positive	mean C_2H_2 production nmol $larva^{-1}$ $hour^{-1}$		standard error of mean
54	39	72	0.347	±	0.127
40*	0	0	0.000	±	0.000

*these larvae were obtained from the Princes Risborough Laboratory (Building Research Laboratory).

3.4 Reticulotermes santinensis

3.4.1 In situ acetylene reduction gave positive results in all experiments.

Table 5. Ethylene production by Reticulitermes Santonensis in situ

		C_2H_4 nmol hour^{-1}	
assay	atmosphere	Per 50 termites	Per g. termite
1	aerobic	0.035	0.301
2	aerobic	0.030	0.294
3	anaerobic	0.047	0.427
4	anaerobic	0.041	0.382

In situ acetylene reduction was also confirmed for samples of termite eaten wood with a mean of 0.167 nmol ethylene formed, hour^{-1} g^{-1} ($\pm$ 0.012).

Enriched wood samples also gave positive acetylene reduction with a mean of 0.595 nmol ethylene formed, hr^{-1} g^{-1} ($\pm$ 0.052).

3.4.2 The additional experiment to investigate the effects of different levels of combined nitrogen and antibiotics on nitrogen fixing activity revealed the following results:

Table 6. Effect of Diet on ethylene production by Reticulitermes santonensis

Diet	C_2H_4 nmol 50 termites^{-1} hr^{-1}
1. Whatman No.2 filter paper + basal salts	0.091
2. + 0.8 mg. $(NH_4)2\ SO_4$	no measurable activity
3. + 8.0 mg. $(NH_4)2\ SO_4$	no measurable activity
4. + 1.2 mg. KNO_3	0.103
5. + 12 mg. KNO_3	no measurable activity
6. + antibiotics	no measurable activity

There was no significant difference in the amount of filter paper consumed by each group of termites.

3.5 Hylotrupes bajulus and Lyctus brunneus

No acetylene reduction could be demonstrated for either of these insects tested in situ or after enrichment. In all 20 larvae of H. bajulus were tested and 45 larvae and 40 adults of L. brunneus.

3.6.1 Of eleven different decaying trees sampled, 5 showed a positive acetylene reduction in situ, whilst 7 showed a positive result after enrichment.

Table 7. Ethylene production in samples of decayed wood both in situ and after enrichment

		mean acetylene dependent ethylene formation nmol g dry wt^{-1} day^{-1}		pH
		in situ	after enrichment	
Sycamore	1	19.20 ± 5.95	*	6.7
	2	4.25 ± 1.61	2.72 ± 0.10 x 10^2	6.3
	3	0.00	0.00	9.1
	4	0.00	2.23 ± 0.42 x 10^2	5.2
	5	0.00	0.72 ± 0.12 x 10^2	4.9
Oak	1	0.00	*	6.2
Willow	1	36.55 ± 3.82	15.81 ± 0.59 x 10^2	4.5
	2	0.00	0.00	6.1
	3	10.21 ± 0.85	5.62 ± 0.42 x 10^2	5.6
Beech	1	0.00	14.65 ± 1.62	5.6
	2	8.24 ± 1.94	4.61 ± 0.36 x 10^2	6.2

*not measured

Samples of sound wood, i.e. showing no visible signs of decay failed to produce any acetylene dependent ethylene both in situ or after enrichment.

3.6.2 Three positive enrichment cultures from decayed wood were investigated to determine the influence of pH on acetylene reduction. The results are shown in tables 8-10 and figures 1-3.

Table 8. Ethylene production from an enriched sycamore culture in relation to pH

	Sycamore sample 2
pH	nmol ethylene formation 1 cm^3 $culture^{-1}$ 24 hrs^{-1}
2.6	4.35
3.0	3.75
3.4	1.06
3.7	0.58
4.3	2.10
4.7	5.58
5.5	22.61
6.0	24.62
6.5	23.24
7.0	23.92
7.5	21.80
8.0	11.42

Table 9. Ethylene production from an enriched beech culture in relation to pH

Beech sample 2

pH	nmol ethylene formation 1 cm^3 $culture^{-1}$ 24 hrs^{-1}
2.6	0.00
2.8	0.00
3.3	1.35
3.6	4.35
4.0	4.25
4.6	5.55
5.1	4.92
5.6	4.28
6.3	4.99
7.0	3.15
7.6	0.00
8.1	0.00

Table 10. Ethylene production from an enriched willow culture in relation to pH

Willow sample 1

pH	ethylene formation 1 cm^3 $culture^{-1}$ 24 hrs^{-1}
2.6	2.36
2.8	1.59
3.3	0.72
3.6	0.00
4.0	2.10
4.6	12.52
5.1	17.41
5.6	17.12
6.3	17.48
7.0	19.52
7.6	27.62
8.0	33.32

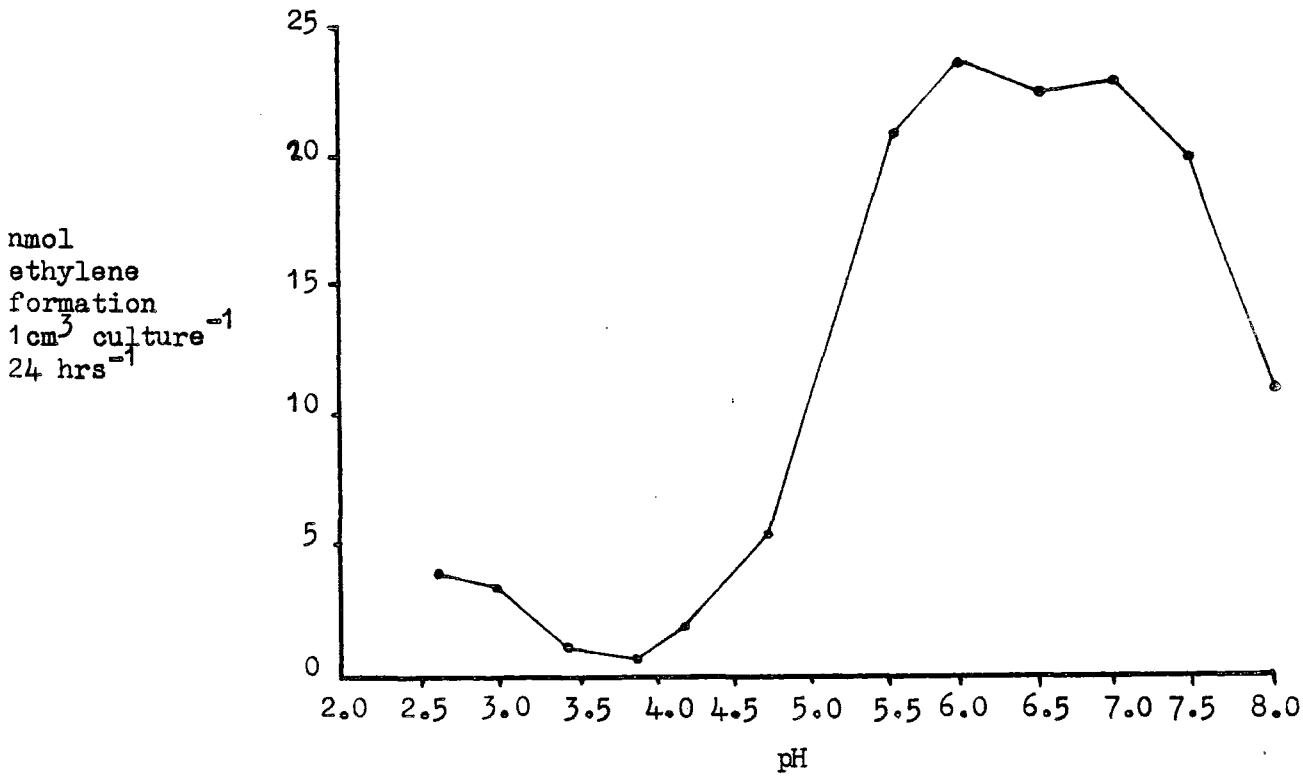

Fig. 1 Ethylene formation from sycamore culture sample 2 incubated at different pH values

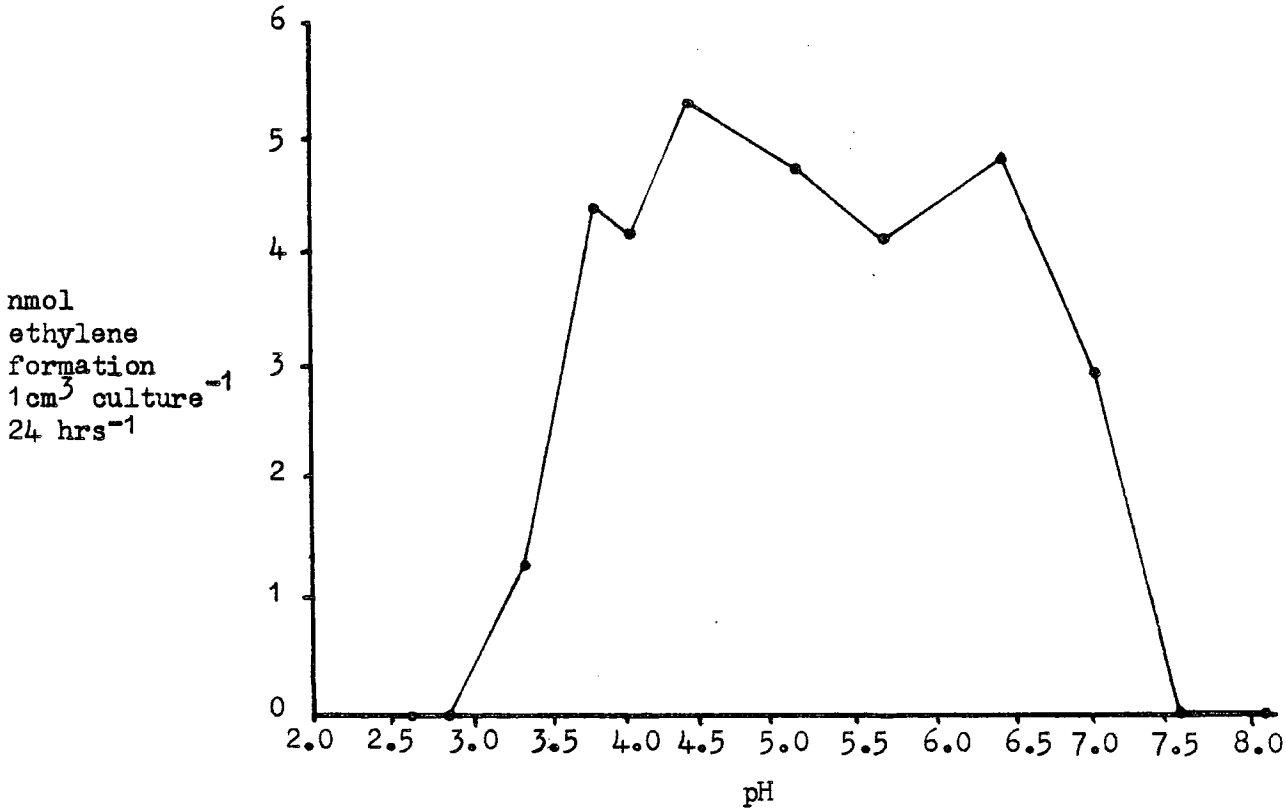

Fig. 2. Ethylene formation from beech culture sample 2 incubated at different pH values

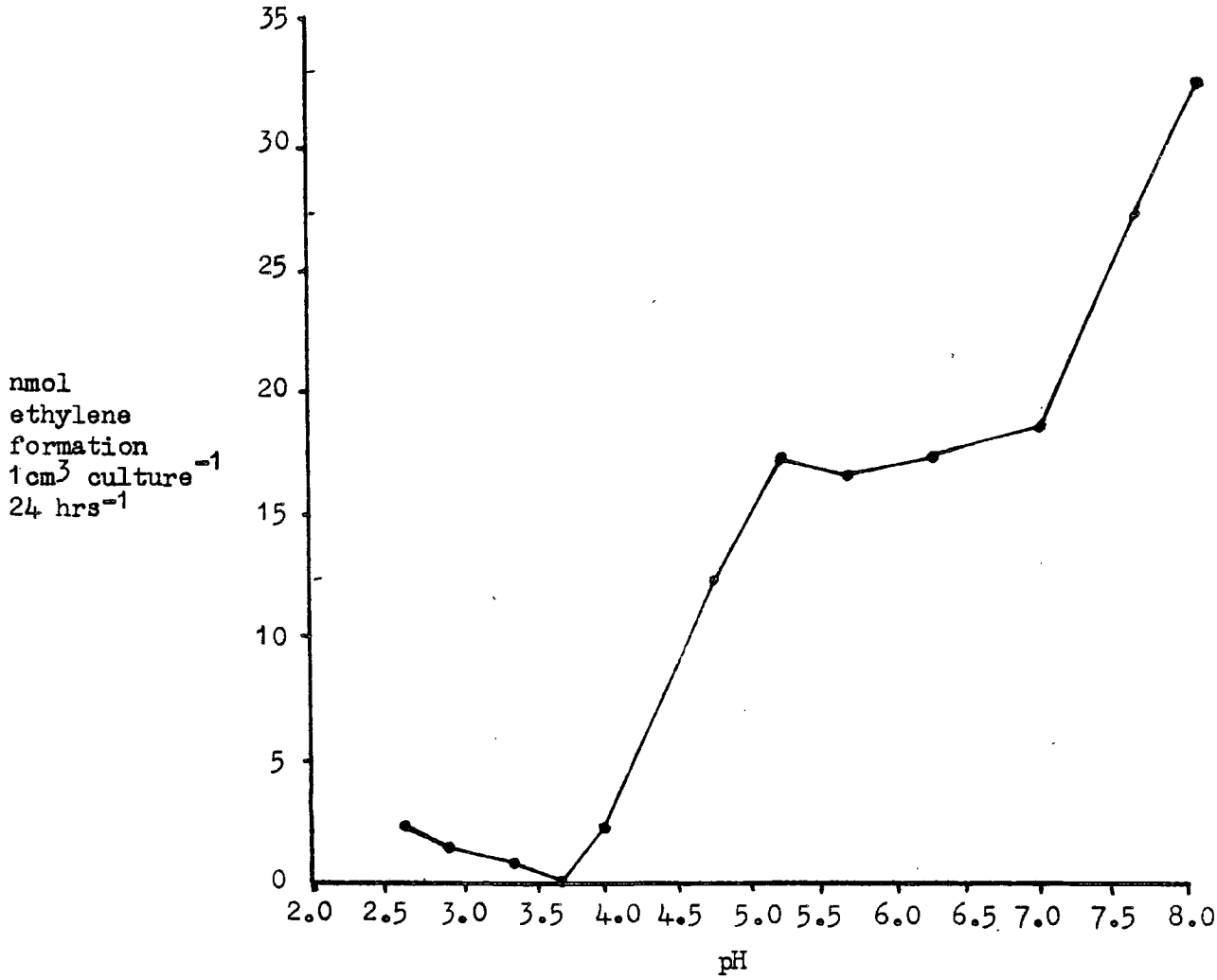

Fig. 3. Ethylene formation from willow culture sample 1 incubated at different pH values

4. DISCUSSION

Although no nitrogen fixing micro-organisms can be demonstrated in the bark beetle Ips cembrae, the possibility exists that they associate with the pine shoot beetle Tomicus piniperda. Overall 19% of T. piniperda larvae gave a positive acetylene reduction, which compared with 79% for the bark beetle larva Dendroctonus terebrans, 22% of D. frontalis emergent adults and 46% of Ips avulsus larvae (Bridges, 1981). Although the number of D. terebrans and T. piniperda larvae tested were comparable, it should be noted that much smaller numbers of D. frontalis (36) and I. avulsus (19) were used in the work reported by Bridges. The number of positive acetylene reduction assays for frass and gallery bark of T. piniperda were 70% and 44% respectively, compared to 100% in similar tests for D. terebrans and I. avulsus, although again fewer samples were tested (Bridges, 1981). The levels of ethylene production by T. piniperda were almost the same after aerobic and anaerobic enrichment (0.048 and 0.040 nmol larva^{-1} hour^{-1}) and were relatively higher than the figures calculated by Bridges of 0.028 nmol larva^{-1} hour^{-1} for D. terebrans larvae. The levels of gallery bark from T. piniperda were 0.015 nmol C_2H_4 g^{-1} hour^{-1}, compared to 0.11 nmol C_2H_4 g^{-1} hour^{-1} calculated for D. terebrans.

The association of nitrogen fixing bacteria with bark beetles has previously been described (Peklo, 1946; Peklo and Satava 1949; Bridges, 1981). However, the number of T. piniperda larvae showing a positive result in the work reported here, may not be high enough to suggest such an association is at least more than a chance occurrence. Due to the low numbers recorded the possibility exists that this insect could have picked up nitrogen fixing bacteria as chance contaminants from the forest ecosystem, as these bacteria are widespread in such systems

(Jones, 1970; Cornaby et al 1973; Aho et al 1974; Larsen et al 1978).

Since no in situ acetylene reduction was demonstrated in the work reported here or by Bridges (1981), it becomes difficult to assess the exact rôle nitrogen fixing bacteria play in such associations. Explanations for the lack of in situ activity would have to include that there was simply not enough nitrogen fixing bacteria present in the larvae to be detected, and after incubation in an enrichment medium they would be selectively enriched to reach detectable levels. One other explanation could be that the enzyme system involved, nitrogenase, had been repressed.

Nitrogen fixing bacteria have their greatest selective advantage in an environment that contains very little available nitrogenous material. Although nitrogenase is a very useful enzyme, only a few species of bacteria possess it, probably because the formation and maintenance of it may be prohibitively uneconomical for most prokaryotic organisms (Brill, 1979). Due to the somewhat unique properties required for nitrogen fixation which can be summarised as

(i) a low reducing potential

(ii) an expensive A.T.P. consumption for each molecule of NH_4^+ formed

(iii) a complex of metalloproteins, with the metals in a unique form

(iv) a mechanism for protecting nitrogenase from oxygen damage,

it could be that the expense of nitrogenase maintenance and production is considerable, which might explain the very efficient cessation of nitrogenase synthesis, when it is no longer required (Gordon et al 1972). Since there is a relatively high level (0.4%) of total nitrogen in pine

inner bark (Hodges et al 1968; Merrill et al 1966) and high levels of combined nitrogen inhibit nitrogenase synthesis (reviewed by Hardy et al 1973), it could be that nitrogenase production had been repressed in these insects, leading to the lack of in situ acetylene reduction.

The fact that nitrogen fixing bacteria have been demonstrated in some larvae of the bark beetle T. piniperda could lead to the tentative suggestion that a symbiotic relationship may exist, although no definite conclusions can be reached about how they influence the insects nitrogen economy. It could be that under certain conditions (weather, tree vigour, overcrowding) such insects do not have enough nitrogen and that nitrogen fixing bacteria could make up the difference. The distribution of nitrogen has been shown possibly to differ with the age of a tree and the time of year (reviewed by Bletchley et al, 1964). Further work (see later) is obviously required on bark beetles, although the original work on D. terebrans has now ceased (J.R. Bridges, personal communication).

The production of ethylene by Anobium punctatum larvae in situ, appears to be the first reported evidence of nitrogen fixation using the acetylene reduction assay. The mean overall production of 0.084 nmol ethylene g. larva^{-1} hour^{-1} compares with a mean production of 0.276 nmol ethylene g. insect^{-1} hour^{-1} for different termite species and castes (Breznak et al, 1973) and 0.040 nmol ethylene g^{-1} day^{-1} for small soil inhabitating animals (Citernesi et al, 1977). The in situ results were confirmed when 72% of larvae incubated in the enrichment medium gave a positive result, with a mean ethylene production of 0.347 nmol larva^{-1} hour^{-1}. Whether this symbiotic relationship is ubiquitous in every infestation by A. punctatum is questionable as the larvae sent from the Princes Risborough laboratory gave negative results both in situ and

after enrichment. A possible reason was that these larvae were maintained in fairly young (approximately 8 year old) hazel blocks and hence would probably have been in a high combined nitrogen environment, which would have repressed nitrogenase production and hence nitrogen fixation.

The extent of an attack by A. punctatum in wood, was thought to depend on its nitrogen content. A direct correlation was also thought to exist between larval survival and growth and quantity of nitrogen in wood, and has been well documented (reviewed by Baker et al, 1970). However it was shown by Baker et al (ibid) that only 25% of nitrogen in wood swallowed by the larvae was retained, and that nitrogen derived from wood failed to meet the demonstrated nitrogen uptake in growing larvae. It was suggested that the simple hypothesis of nitrogen being a limiting factor could not be the entire explanation.

It would seem, from the results reported here, that A. punctatum larvae are able to obtain extra nitrogen from a symbiotic relationship with nitrogen fixing bacteria, living somewhere within their tissues. The implications of how this effects the nitrogen economy of the larvae and their ultimate success and survival in colonizing wood can only be surmised, but with such a relationship the larvae may never be short of nitrogen, even in wood low in nitrogen. The extent of such a symbiotic relationship and the consequent levels of nitrogen fixed, may even explain why the larval stage is so variable in duration. The undoubted limiting effect of nitrogen in wood may be due to specific needs for essential amino-acids or trace components which vary with nitrogen content (Baker, 1970). The relevance of nitrogen fixation to the survival of the insect may even have economic implications, and it is interesting to speculate that if fixation is indispensible to its survival, eradication

measures could be directed towards destroying the fixation system in these insects.

The in situ results for the termite Reticulitermes santonensis indicated ethylene production, hence nitrogen fixation, for both aerobic and anaerobic atmospheres. Under aerobiosis there was a mean of 0.297 nmol ethylene $hour^{-1}$ g^{-1}, whilst under anaerobiosis there was 0.404 nmol ethylene $hour^{-1}$ g^{-1}. These results are compatible with those for another termite genus, Kalotermes minor, which showed about equal ethylene formation, under different atmospheric conditions (Benemann, 1973). They also compare with another species of the same genus, R. flavipes, which produced 0.204 nmol ethylene $hour^{-1}$ g^{-1} under aerobic conditions and with 0.695 nmol ethylene $hour^{-1}$ g^{-1} for Coptotermes formosanus (Breznak et al, 1973). Nitrogen fixing bacteria were also found in the wood which had been colonized by the termites, producing 0.167 nmol ethylene $hour^{-1}$ g^{-1} in situ and 0.595 nmol ethylene $hour^{-1}$ g^{-1} after enrichment.

Nitrogen fixing bacteria have been found to exist in several species of termite (Benemann, 1973; Breznak et al, 1973) and their presence in R. santonensis can now be included. It is beyond the scope of this present work to quantify the rôle of nitrogen fixing bacteria in the nitrogen balance of termites. Conversion factors for different nitrogen fixing bacteria and systems, which convert acetylene reduced to nitrogen, have been listed by Hardy et al (1973). However since there are no data on the nitrogen content of the termite under current investigation and no bacterial isolates were identified it was considered a task perhaps best suited for future investigation. It is relevant to note that Benemann (1973) concluded from studies on Kalotermes, that it would take 30 months to double the nitrogen content of the termites, using the

highest observed rates of nitrogen fixation and on average 7-8 years. Nevertheless the presence of nitrogen fixing bacteria in R. santonensis and other genera of termites, could mean they play an important rôle in the soil, by adding combined nitrogen to it and hence increasing soil fertility.

The levels of ethylene produced by termites fed on filter paper containing no added nitrogen source was 0.091 nmol 50 termites^{-1} hour^{-1}, which was more than double that produced by the termites fed on wood. In a comparable study with C. formosanus, the termites produced more than ten times the amount of ethylene when fed on filter paper with no added nitrogen, compared to a wood diet (Breznak et al, 1973). A possible explanation would be that presumably filter discs contain less combined nitrogen than the wood consumed by the termites, so that there would be less repression of the nitrogenase enzyme.

The addition of NH_4^+ at both concentration of 8.0 mg. and 0.8 mg. completely abolished all detectable acetylene reduction and hence nitrogen fixation. This was perhaps not surprising since ammonium ions are known to repress nitrogenase synthesis because they are preferred to nitrogen (Strandberg et al, 1968). The presence of 12 mg. of KNO_3 also completely abolished acetylene reduction, but the lowest concentration of 1.2 mg. appeared to have no effect at all, with an ethylene production of 0.103 nmol 50 termites^{-1} hour^{-1}. In a study by Breznak et al (1973) using C. formosanus additions of NH_4^+ or NO_3^- to filter paper diminished acetylene reduction to an extent which varied with the amount of nitrogen compound impregnated on the filter paper, but never completely abolished it. It has been demonstrated that NO_3^- alone is not capable of repressing nitrogenase (Sorger, 1969), and it

is thought that because NO_3^- easily converts to NH_4^+ by the enzyme nitrate reductase, any repression by NO_3^- may merely manifest an accumulation of enough internal NH_4^+ to repress nitrogenase synthesis (Brill, 1979). It could be that at a concentration of 1.2 mg. there was not enough nitrate to be converted to NH_4^+, hence the level of acetylene reduction observed. When the termites were fed filter paper impregnated with antibacterial anti-biotics, all detectable acetylene reducing activity was abolished. This result is consistent with the idea that bacteria in the termite gut are responsible for nitrogen fixation, and hence would be inhibited by the antibiotics.

The lack of any acetylene reduction both in situ and after enrichment in both Hylotrupes bajulus and Lyctus brunneus indicates a lack of any symbiotic relationship with nitrogen fixing bacteria. Since there appears to be no record of any gut symbionts being detected in either of these insects, perhaps the results are not surprising.

The short-term study on nitrogen fixing activity in decayed wood revealed in situ acetylene reduction in 5 of the 11 different trees sampled, with ethylene production ranging from 4.25 to 36.55 nmol g dry wt^{-1} day^{-1}. The pH range of these samples was 4.5 to 6.7. These results compare with rates in chestnut logs showing early decay of 24.78 nmol ethylene g dry wt^{-1} day^{-1} to 36.79 nmol ethylene g dry wt^{-1} day^{-1} in logs showing signs of late decay and had a pH range of 3.2 to 4.1 (Cornaby et al, 1972). Larsen et al (1979) found ethylene production in a range of 1.38 - 10.55 nmol g dry wt^{-1} day^{-1} for samples of decayed Western Hemlock, Subalpine Fir and Douglas Fir. Preliminary tests with decayed heartwood blocks of white fir trees produced 142 nmol ethylene day^{-1} in samples 20 cm. diameter and 20 cm. long although these estimates were not related to weight of sample (Aho et al, 1974).

Calculations have since been carried out on this data and revealed levels of 0.3 nmol ethylene g dry wt^{-1} day^{-1}, which appears rather low (Larsen et al, 1979).

There was no sample that showed acetylene reduction in situ and not after enrichment, but three samples showed reduction after enrichment and gave a negative result in situ. It can only be presumed that in these cases the quantity of nitrogen fixing bacteria was too low to be detected until enrichment. Two samples were negative both in situ and after enrichment. The levels of ethylene production after enrichment ranged from 0.146×10^2 to 15.81×10^2 nmol g dry wt^{-1} day^{-1}. These levels compare with those after enrichment of 4.96 to 95.07×10^2 nmol $culture^{-1}$ day^{-1} in white fir trees (Aho et al, 1974) and 0.012×10^2 to 18.14×10^2 nmol g wet wt^{-1} day^{-1} for beech and oak (Sharp et al, 1973).

This current work, although of a preliminary nature, may go some small way in providing more evidence for the presence of nitrogen fixing micro-organisms in the ecology of deteriorating and decaying wood. They indicate that nitrogen fixing micro-organisms colonize some but not all decaying wood sites and could probably provide additional nitrogen for decay organisms and hence play a rôle in regulating the rate of wood decomposition in forest ecosystems. It may be that in conditions of very low nitrogen, pioneer colonizing fungi are able to utilize this additional nitrogen (Sharp, 1974). Aho et al (1974) suggested that a sort of mutualism existed between bacteria and fungi, the bacteria providing the nitrogen necessary for the synthesis of fungal wood digesting enzymes, whilst the fungi provided cellulose and hemi-cellulose breakdown together with the formation of organic acids. Although Sharp (1975) failed to demonstrate

the transfer of labelled ^{15}N from wood into fungi, Larsen et al (1979) found the highest rates in brown rot decay systems which were known to be an important ecological niche for the formation of mycorrhizae and hence suggested a strong nutritional relationship existed between mycorrhizae and nitrogen fixation.

Since no acetylene dependent ethylene and therefore evidence of nitrogen fixation was associated with sound wood (i.e. wood showing no signs of decay) these results suggest that decaying wood could be a useful substrate for colonization by nitrogen fixing bacteria. It is difficult, however, to suggest what makes a particular piece of decayed wood the ideal environment for colonization, especially since some decayed samples had no nitrogen fixing bacteria at all. There may be a demand for certain growth requirements such as: the presence of relatively high levels of carbon and very low levels of combined nitrogen so that carbon:nitrogen ratios are very high; the presence of adequate growth factors like Mo or Fe; the degree and state the decay has reached; the necessity for an optimum pH level; a lack of other inhibitory micro-organisms, or inhibitory substances in the wood.

The results of the experiments to investigate a possible optimum pH for nitrogen fixation in wood, failed to reveal any single optimum (see figures 1-3). The culture obtained from the sycamore sample showed an optimum at pH 6.0; the beech culture showed a broad range of production between pH 3.6 and 6.3, whilst the willow sample showed an optimum at pH 8.0. This compares to the results of Sharp (1974) who found a single optimum of pH 3.5 for beech veneers. Hardy et al (1971) reported that nitrogenase had a pH optimum of between 6 to 8, whilst Stewart (1966) reported a neutral pH to be favoured in a variety of different ecosystems. It can only be concluded that in this current

work, the enriched cultures obtained from the wood, providad such a mixed culture, that differing pH optima resulted from the different micro-organisms present. It is perhaps interesting to note that in all the samples tested, except one, the pH of the wood was acidic, and that the sample with the lowest pH (4.5) had the highest level of ethylene production (see table 7).

5. SUGGESTIONS FOR FURTHER WORK

The scope for further work from such a preliminary study of nitrogen fixation in wood boring insects and decayed wood is considerable. There are over sixty species of bark beetle on the British list, occurring on twenty-eight different hosts including both conifers and hardwoods. Therefore it would appear there is enough material to continue investigations and determine whether a symbiotic relationship between such insects and nitrogen fixing bacteria is widespread or just a chance occurrence in a few species.

In this current study, due to its short-term nature, no attempt was made to isolate and identify bacteria from the insects examined. This would involve serially diluting cultures that reduced acetylene and plating out onto a solid enrichment medium, before picking out individual colonies and isolating on streak plates of an enrichment medium to obtain pure strains. Biochemical tests could then be carried out on such cultures to characterise and name the bacteria concerned. Attempts could also be made to quantify the numbers of bacteria concerned using most probable number techniques to investigate whether a lack of in situ acetylene reduction can be attributed to low levels of nitrogen fixing bacteria.

One important piece of work not carried out in this study, again due to the time factor, was to determine levels of combined nitrogen in the wood. It has been speculated, in this study, that if nitrogenase activity was repressed, the cause may have been high levels of combined nitrogen. Levels of nitrogen in both infested wood, and indeed the decayed wood, could be checked using Kjeldahl techniques and related to the occurrence of nitrogen fixing bacteria.

The occurrence of nitrogen fixing micro-organisms in Anobium

punctatum needs further investigation. This could involve isolating larvae from infested wood and attempting to correlate acetylene reduction activity with nitrogen levels in the wood. Since Anobium is such an economically important insect it would also be interesting to discover the exact site of the symbiotic bacteria and also determine how such symbionts become established in the larval stage.

The role of nitrogen fixing bacteria in decaying wood also merits further attention. It may involve experiments which seek to determine what factors restrict or somehow prevent nitrogen fixing bacteria from colonizing decaying wood. It should be possible to introduce antibacterial antibiotics and then investigate the success of decay fungi without the extra input of nitrogen provided by nitrogen fixation. It may also be possible to determine the total nitrogen fixation rates in decaying wood for a particular ecosystem to determine the contribution made by fixation in the overall nitrogen budget.

Finally, the question of why and how decaying wood is colonized by nitrogen fixing micro-organisms also could be investigated. A much greater survey of decayed wood needs to be carried out to determine the extent of colonization by nitrogen fixing bacteria. This could also involve investigating parameters additional to the ones reported in this work, e.g. the state and type of rot involved, moisture contents need to be determined as acetylene penetration may differ between samples with variable moisture contents so influencing the concentration of acetylene available for reduction; levels of combined nitrogen; effects of ambient temperature.

6. SUMMARY

1. The possibility that a symbiotic relationship exists between nitrogen fixing bacteria and wood boring insects was investigated using the acetylene reduction assay.

2. Although no in situ acetylene (nitrogen) reduction could be detected in the larvae of the pine shoot beetle Tomicus piniperda, 20% of the larvae tested showed acetylene reduction after incubation in an enrichment medium. Mean levels of 0.048 nmol ethylene larva^{-1} hr^{-1} and 0.040 nmol ethylene larva^{-1} hr^{-1} were recorded aerobically and anaerobically respectively. Samples of insect frass and gallery bark also showed positive acetylene (nitrogen) reduction, after enrichment.

3. No acetylene reduction could be detected for the bark beetle Ips cembrae, testing both in situ and after enrichment.

4. Positive acetylene (nitrogen) reduction was shown in the common furniture beetle larvae Anobium punctatum. In situ levels of 0.084 nmol ethylene g larva^{-1} hr^{-1} were recorded, whilst 72% of the larvae showed positive acetylene reduction, after enrichment, with a mean level of 0.347 nmol ethylene larva^{-1} hr^{-1}. This appears to be the first reported evidence of nitrogen fixation using the acetylene reduction assay, in this economically important insect.

5. The presence of nitrogen fixing bacteria were also demonstrated in the termite Reticulitermes santonensis, giving a positive acetylene (nitrogen) reduction in situ of 0.279 nmol ethylene g^{-1} hr^{-1} (aerobically) and 0.404 nmol ethylene g^{-1} hr^{-1} (anaerobically). Samples of termite infested wood also showed positive acetylene reduction.

6. The levels of ethylene production for the same termite fed on filter paper, with no added nitrogen, was 0.091 nmol 50 termites^{-1} hr^{-1} which was more than double that found when the insects were fed on a diet of wood. With a diet of paper plus mineral salts and different concentrations of NH_4^+, all detectable acetylene (nitrogen) reduction disappeared. High levels (12 mg.) of NO_3^- also inhibited reduction, but low levels (1.2 mg.) had no effect. With a diet of filter paper impregnated with antibacterial antibiotics, all detectable acetylene reduction ceased.

7. No acetylene (nitrogen) reduction could be detected in the wood boring insects Hylotrupes bajulus and Lyctus brunneus.

8. Samples of decaying wood were investigated using the same techniques. Of eleven different sites tested, five showed in situ levels of acetylene (nitrogen) reduction due to nitrogen fixing micro-organisms, with ethylene production ranging from 4.25 to 36.55 nmol g dry wt^{-1} day^{-1}. All these samples gave positive results after enrichment in addition to three samples which had given negative results in situ. Three samples gave negative results both in situ and after enrichment.

9. Enriched cultures from the decaying wood samples showing a positive acetylene (nitrogen) reduction were incubated at different pH values. A sycamore culture showed an optimum at pH 6.0; a beech culture showed a broad range of maximum ethylene production between pH 3.6 to 6.3; a willow sample showed an optimum at pH 8.0.

ACKNOWLEDGEMENTS

I wish to thank Mr. G. Banbury of the University of Durham for all his help, advice and optimism given throughout this project.

I also wish to acknowledge the help and efficacy of the technical staff in the Botany Department whose assistance was most appreciated.

Finally, I would also like to thank Durham County Council Education Department and the staff at New College Durham for allowing me a year's secondment to return to study.

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